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Lyn inhibits osteoclast differentiation by interfering with PLCγ1-mediated Ca^{2+} signaling

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ABSTRACT

Osteoclasts differentiate from macrophage-lineage cells to become specialized for bone resorption function. By a proteomics approach, we found that Lyn was down-regulated by the osteoclast differentiation factor, receptor activator of NF-κB ligand (RANKL). The forced reduction of Lyn caused a striking increase in the RANKL-induced PLCγ1, Ca^{2+}, and NFATc1 responses during differentiation. These data suggest that Lyn plays a negative role in osteoclastogenesis by interfering with the PLCγ1-mediated Ca^{2+} signaling that leads to NFATc1 activation. Consistent with the in vitro results, in vivo injection of Lyn specific siRNA into mice calvariae provoked a fulminant bone resorption. Our study provides the first evidence of the involvement of Lyn in the negative regulation of osteoclastogenesis by RANKL.

1. Introduction

Osteoclasts are multinucleated giant cells that are responsible for bone resorption. Deregulation of osteoclast number and activity leads to bone diseases such as osteoporosis and osteopetrosis. Therefore, it is important to identify new molecular targets that regulate osteoclast differentiation or function for therapeutic purposes. Receptor activator of NF-κB ligand (RANKL) is the essential factor for osteoclastic differentiation while macrophage colony stimulating factor (M-CSF) is crucial for precursor proliferation and cell survival during the differentiation process [1].

The binding of RANKL to its receptor RANK, a member of tumor necrosis factor receptor (TNFR) superfamily, initiates activation of signaling pathways that involve recruitment of TNF receptor associated factor (TRAF) family proteins and subsequent induction of the nuclear factor of activated T cells c1 (NFATc1) and other osteoclastogenic genes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K. NFATc1 is a calcineurin- and Ca^{2+}-regulated transcription factor. Calcium stimulates calcineurin-mediated dephosphorylation of NFATc1 and consequent translocation of NFATc1 to the nucleus. The calcium signals required for osteoclastogenesis may be attained by activation of PLCγ [2]. Activated PLCγ hydrolyzes PIP_2 to inositol 3-phosphate (IP_3) that increases intracellular Ca^{2+} and thereby stimulates NFATc1 activation. Induction of NFATc1 is enhanced by its autoamplificatory property.

Lyn, a member of the Src family tyrosine kinase, plays a pivotal role in ligand-induced signaling in B-lineage lymphoid cells [3]. In B lymphocytes, Lyn phosphorylates the immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which facilitates the recruitment of protein tyrosine phosphatase SHP-1 [4]. Whether Lyn plays a role in osteoclastogenesis has not been investigated to date.

By a proteomic analysis, we found regulation of Lyn expression by RANKL. We further characterized the role of Lyn in osteoclast differentiation. Our results indicate that Lyn has a function in counterbalancing osteoclastogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Anti-Lyn and anti-NFATc1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). Human soluble RANKL and M-CSF were purchased from Pepro-Tech (Rocky Hill, NJ). Lipofectamine 2000 was from Invitrogen Life Technologies (Carlsbad, CA). Anti-actin antibody and all other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Bone marrow-derived macrophage culture and osteoclast differentiation

Bone marrow-derived macrophages (BMMs) were generated as described previously [5]. BMMs were cultured in α-MEM

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containing 10% FBS, 30 ng/ml M-CSF, and 200 ng/ml RANKL for 5 days to generate osteoclasts. Preparation of calvarial osteoblasts was performed as described [6]. For coculture, calvarial osteoblasts and BMMs were cultured in the presence of vitamin D_3 (10^{-8} M) and PGE_2 (10^{-6} M) for 6 days. For osteoclast differentiation from whole bone marrow cells (WBM), cells were flushed out of the bone marrow cavities with α-MEM. WBM were plated at 1×10^6 cells per well in 48-well plates and cultured with vitamin D_3 (10^{-8} M) for 8 days.

2.3. Cell-surface biotinylation and LC/MS/MS experiments

BMMs were surface biotinylated using Pinpoint cell-surface protein isolation kit (Pierce, Rockford, IL) following the manufacturer's instruction and LC/MS/MS analysis was performed as described previously [7].

2.4. Plasmid construct

The entire coding region of the mouse Lyn cDNA was PCR-amplified from reverse-transcribed mouse macrophage cDNA with primers 5′-aggatccaccatgggatgtattaaatca-3′ and 5′-actcgagctacggttgctgctgatac-3′. Amplified PCR product was digested with BamHI/XhoI and cloned into pCMV2B vector.

2.5. Gene knock-down by oligonucleotide siRNA

The 22-nucleotide small interfering RNA (siRNA) duplexes for Lyn and negative control were purchased from Invitrogen Life Technologies (Carlsbad, CA). For siRNA transfection, BMMs were seeded in 48-well plates at a density of 3×10^4 cells/well. Twenty-four hours later, 20 pmoles of oligonucleotide siRNA duplexes were mixed with 0.5 μl of Lipofectamine 2000 in 200 μl DMEM and transfected into the cells for 4 h following the manufacturer's instruction.

2.6. Transfection of RAW 264.7

RAW 264.7 cells were seeded in 48-well plates at 1×10^4 cells/well. Cells were transfected with 0.4 μg of pCMV2B-Lyn or pCMV2B plasmids mixed with 1 μl Lipofectamine 2000 and 200 μl DMEM for 4 h following the manufacturer's instruction.

2.7. RT-PCR

Total RNA was prepared using Trizol (Invitrogen) and cDNAs were synthesized from 1 μg of RNA using SuperScript II reverse transcriptase (Invitrogen). The PCR primer sequences used are as follows: Lyn, 5′-tagaagagcatggggaatgg-3′ (forward) and 5′-gaaagctcctgcactgttcc-3′ (reverse); TRAP, 5′-acttccccagcccttactaccg-3′ (forward) and 5′-tcagcacatagcccacaccg-3′ (reverse).

2.8. Western blotting

Cells were lysed in RIPA buffer containing protease inhibitors. Whole cell extracts were separated on polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated overnight at 4 °C with specific primary antibodies. The membrane was washed, incubated for 1 h with a secondary antibody-HRP conjugate, and developed using an enhanced chemiluminescence system.

2.9. Measurement of intracellular Ca^{2+} concentrations and oscillation

For measurement of total intracellular Ca^{2+} concentrations, cells cultured on plates were loaded with Fluo-4 NW dye solution (Molecular Probes, Eugene, OR) at 37 °C for 30 min and then incubated at room temperature for an additional 30 min. The CytoFluor plate reader (Applied Biosystems, Foster City, CA) was used for detection with an excitation/emission filter pair (485/535 nm). To monitor the Ca^{2+} oscillation response in individual cells, cells cultured on cover slips were loaded with Fura-2/AM and the assay was performed as previously described [8].

2.10. In vitro pit formation assay

siRNA-transfected BMMs were cultured on dentin slices for 5 days in the presence of RANKL and M-CSF. The cells were removed by sonication and the dentin slices were stained with 1% toluidine blue and the bone resorption area was determined with image analysis software (Image Pro-Plus, Media Cybernetics).

2.11. TRAP staining

Cells were fixed with 3.7% formaldehyde. After washing with PBS, cells were incubated with 0.1% Triton X-100 for 5 min. Cells were washed again and stained for TRAP activity for 40 min at 37 °C in dark using the Leukocyte Acid Phosphatase Assay kit (Sigma) following the manufacturer's instruction. TRAP staining of mice calvariae was also performed in the same way.

2.12. Calvarial bone resorption assay

Thirty microliters of 20 μM Lyn specific siRNA or scrambled control siRNA were mixed with 10 μl of Lipofectamine 2000 and injected onto calvariae three times with 2-day intervals. One day after the first injection, collagen sheet soaked with 10 μg of RANKL or PBS and implanted into the center of calvariae. Mice calvariae (5 per group) were collected and micro-computed tomography (μ-CT) was performed with 1072 Microtomograph (SkyScan, Belgium). Histological sections were generated after decalcification and embedding into paraffin and the sections were stained for TRAP activity. To evaluate in vivo gene silencing by Lyn siRNA, total RNA was extracted from calvariae and RT-PCR analyses were performed.

3. Results

In an attempt to discover new molecules involved in the regulation of osteoclast differentiation, the profile of membrane proteins expressed in macrophages and osteoclasts was compared. Following cell surface biotinylation of bone marrow-derived macrophages (BMMs) cultured with or without RANKL for 36 h, biotinylated proteins were isolated and subjected to SDS–PAGE. As shown in Fig. 1A, several protein bands showed differential expression upon RANKL treatment. Those protein bands were cut out and processed for liquid chromatography followed by tandem mass spectrometry. We identified a protein from ~55 kDa band of which density apparently decreased by RANKL as Lyn (Fig. 1B and C). To confirm the reduced expression of Lyn by RANKL, BMMs treated with or without RANKL in the presence of M-CSF were subjected to RT-PCR and Western blotting. Fig. 1D shows that Lyn mRNA and protein levels were decreased by RANKL treatment for 24 h. Next, we investigated whether the activity of Lyn may be regulated by RANKL. Since tyrosine phosphorylation of Lyn correlates well with the catalytic activity of Lyn, we measured Lyn tyrosine phosphorylation in BMMs stimulated with RANKL plus M-CSF for 30 min. As shown in Fig. 1E, the tyrosine phosphorylation of the 53 and 56 kDa bands of Lyn immunoprecipitates increased upon stimulation. This result indicates that the catalytic activity of Lyn is stimulated by osteoclastogenic signal.

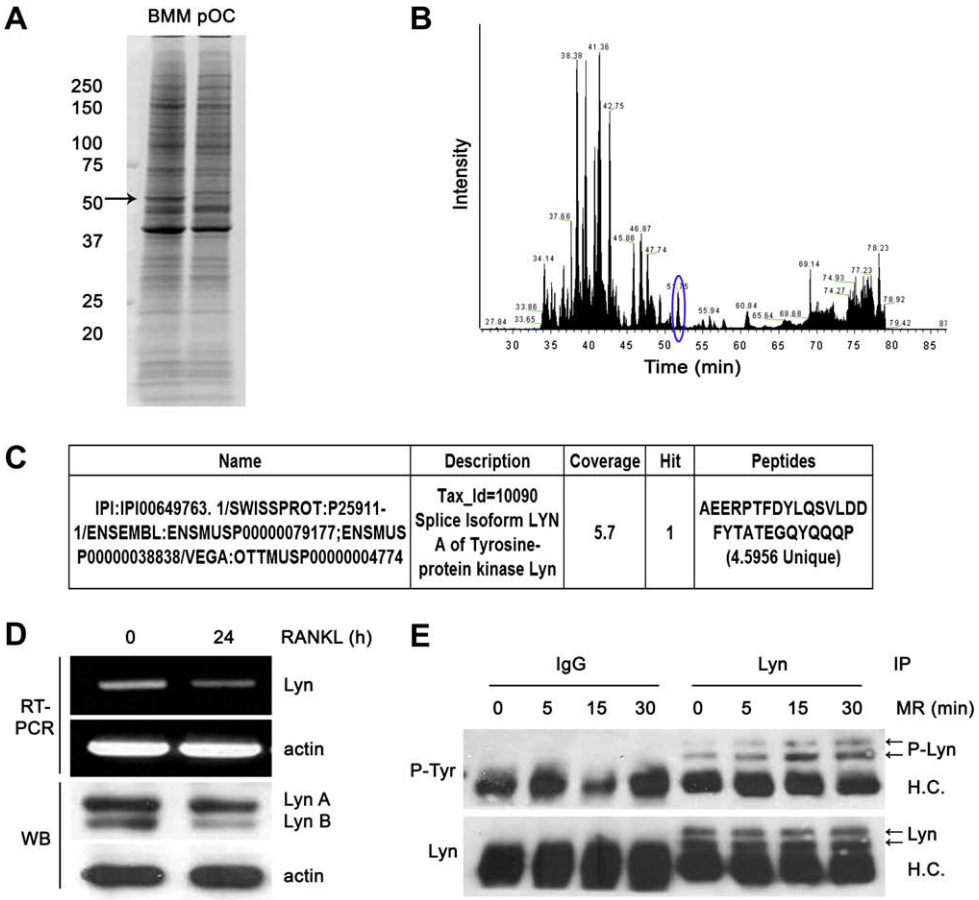

Fig. 1. Proteomic analysis of Lyn expression on osteoclasts. (A) BMMs were incubated with 30 ng/ml M-CSF plus 200 ng/ml RANKL for 36 h followed by biotinylation of surface proteins. The biotinylated proteins were separated on an 8–16% SDS gel. The protein bands were visualized by staining with SYPRO Ruby. (B) The protein band indicated by an arrow in (A) was digested with trypsin and subjected to LC/MS/MS analysis. The profile of peptides eluted from LC is shown. The peak indicated with a circle was identified as a peptide of Lyn. (C) The sequence of the Lyn peptide obtained by mass spectrometry is presented. (D) BMMs were cultured with 30 ng/ml M-CSF plus 200 ng/ml RANKL for 24 h. The expression levels of Lyn mRNA and protein were determined by RT-PCR and Western blotting. (E) Whole cell extracts from RANKL stimulated BMMs were immunoprecipitated with control rabbit IgG or Lyn antibody followed by Western blotting using phospho-tyrosine or Lyn antibody. HC: heavy chain of antibody.

As the expression level of Lyn was decreased by RANKL treatment, we investigated the functional role of Lyn for osteoclastogenesis by gene knock-down. BMMs were transfected with Lyn specific siRNA or scrambled control siRNA oligonucleotide duplexes. The efficiency of gene silencing was assessed by RT-PCR and Western blotting. Both the mRNA and protein levels of Lyn were decreased in Lyn siRNA-transfected BMMs compared with scrambled siRNA-transfected cells (Fig. 2A). Next, to evaluate the effect of Lyn silencing on osteoclastogenesis, the siRNA-transfected BMMs were cultured with RANKL plus M-CSF for 5 days and stained for TRAP activity. The number of $TRAP^+$ osteoclasts generated from Lyn siRNA-transfected BMMs was strikingly higher than that from control cells (Fig. 2B, top panel). When the siRNA-transfected BMMs were induced to differentiate by coculturing with calvarial osteoblasts, similar results were obtained (Fig. 2B, middle panel). Furthermore, the pro-osteoclastogenic effect of Lyn knock-down was also evident when whole bone marrows cells were transfected with siRNA and osteoclastogenesis was induced (Fig. 2B, bottom panel). We also assessed the effect of Lyn silencing on osteoclastogenesis by evaluating the resorption activity of osteoclasts generated from Lyn silenced or control siRNA-transfected BMMs in vitro. As shown in Fig. 2C, the area of resorption pits produced on dentine slices with Lyn silenced-BMMs was greater than that with control siRNA-transfected cells. Next, we examined the effect of Lyn overexpression on osteoclastogenesis. RAW 264.7 cells were transfected with Lyn or a control plasmid and cultured with RANKL. Lyn overexpression resulted in an evident decrease in the number of osteoclasts generated (Fig. 2D). All of these results demonstrate that Lyn plays a negative role for osteoclast differentiation.

To gain molecular insights into the suppressive role of Lyn in osteoclastogenesis, we investigated the effect of Lyn silencing on RANKL-induced signaling pathways. MAPK signaling cascade is one of the well-characterized pathways activated by RANKL. Therefore, we assessed the activation of MAPKs (p38, Erk1/2, JNK) in Lyn silenced-BMMs. There was no significant difference in the phosphorylation of MAPKs between Lyn siRNA- and control siRNA-transfected cells upon RANKL stimulation (data not shown). On the other hand, phosphorylation of PLCγ1 was conspicuously enhanced in Lyn silenced-BMMs compared with control cells (Fig. 3A). PLCγ activation leads to generation of IP_3 that evokes Ca^{2+} signal, which is required for activation and induction of NFATc1 in osteoclasts [2]. Therefore, we examined the RANKL-induced Ca^{2+} signal in Lyn silenced-BMMs, which is crucial for autoamplification of NFATc1 during osteolcast differentiation. As shown in Fig. 3B, the total intracellular Ca^{2+} concentration was elevated in Lyn silenced-BMMs compared with scrambled siRNA-transfected cells. Furthermore, the amplitude and frequency of Ca^{2+} peaks in the oscillation response of individual cells were also significantly higher in Lyn siRNA-transfected cells (Fig. 3C). To examine the effect of increased Ca^{2+} signaling by Lyn depletion on NFATc1, we assessed the expression level of NFATc1 in Lyn si-

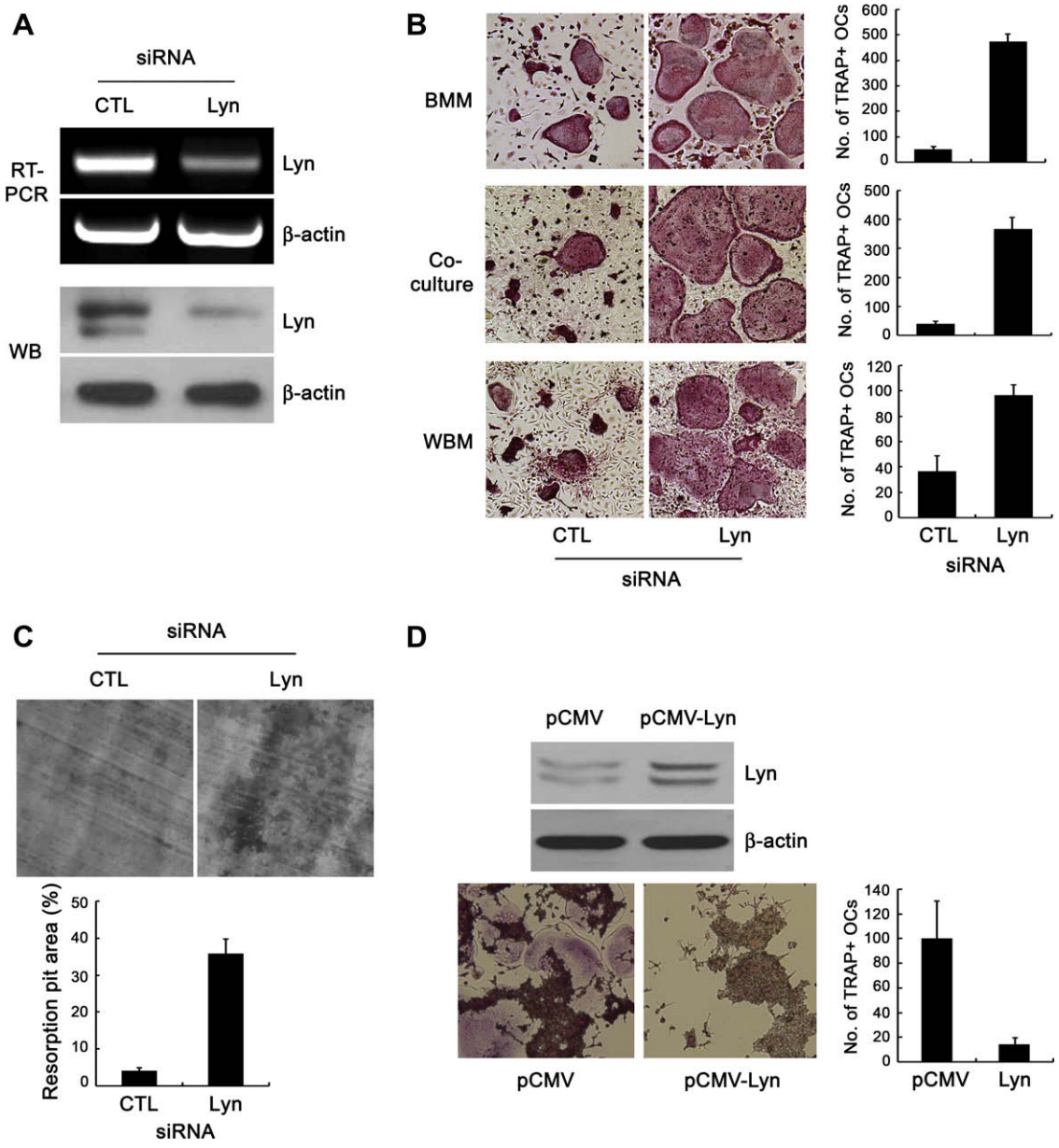

Fig. 2. Effect of Lyn silencing on osteoclast differentiation. (A) BMMs were transfected with scrambled siRNA or Lyn siRNA. After 24 h, the levels of Lyn mRNA and protein were analyzed. (B) siRNA-transfected BMMs were incubated with RANKL (top panel) or cocultured with calvarial osteoblasts (middle panel) for 5 days and then TRAP stained. siRNA transfected whole bone marrow cells (WBM) were differentiated for 8 days in the presence of vitamin D_3 and then TRAP stained (bottom panel). The number of $TRAP^+$ multinucleated cells was scored. (C) Lyn or control siRNA-transfected BMMs were placed on dentine slices and cultured in the presence of RANKL for 5 days. The resorbed pit area was assessed after removing cells and staining with toluidin blue. (D) RAW 264.7 cells were transfected with pCMV or pCMV-Lyn. After 24 h, the cells were cultured with RNAKL for 3 days and then TRAP stained. The number of $TRAP^+$ multinucleated cells was scored.

lenced-BMMs. As shown in Fig. 3D, NFATc1 level was much higher in Lyn siRNA-transfected cells compared to the control siRNA-transfected cells. Taken together, these results suggest that Lyn inhibits RANKL-induced NFATc1 activation by suppressing PLCγ-mediated Ca^{2+} oscillation during osteoclast differentiation.

Next, we examined upstream events of the Lyn-mediated calcium signaling. c-Src is a molecule reported to be crucial to calcium signaling for osteoclastogenesis and Syk serves a substrate of c-Src kinase in the calcium signaling cascade [9]. We found that the c-Src phosphorylation induced by RANKL stimulation was greater in Lyn silenced-BMMs (Fig. 3E). Furthermore, Lyn was co-immunoprecipitated with Syk (Fig. 3F). These results suggest that Lyn may regulate osteoclastogenesis by restraining the c-Src-mediated calcium signaling.

With the in vitro evidence that Lyn plays a negative function in osteoclastogenesis, we next evaluated the role of Lyn in vivo. Mice calvariae were implanted with collagen sheets soaked with either RANKL or PBS. Lyn specific siRNA or control scrambled siRNA was injected into the calvariae trice with a 2-day interval and stained for TRAP activity. As shown in Fig 4A, Lyn mRNA level was lower in Lyn siRNA-injected calvariae than compared with that in control siRNA-injected mice. We also observed elevated TRAP mRNA levels in Lyn silenced calvariae (Fig. 4A). Furthermore, calvariae of Lyn siRNA-injected mice revealed an increased number of TRAP-positive osteoclasts compared with the control siRNA-injected mice both in RANKL and PBS administration conditions (Fig. 4B). Consistently, μ-CT analysis showed a significantly reduced bone volume in Lyn siRNA-injected calvariae compared with

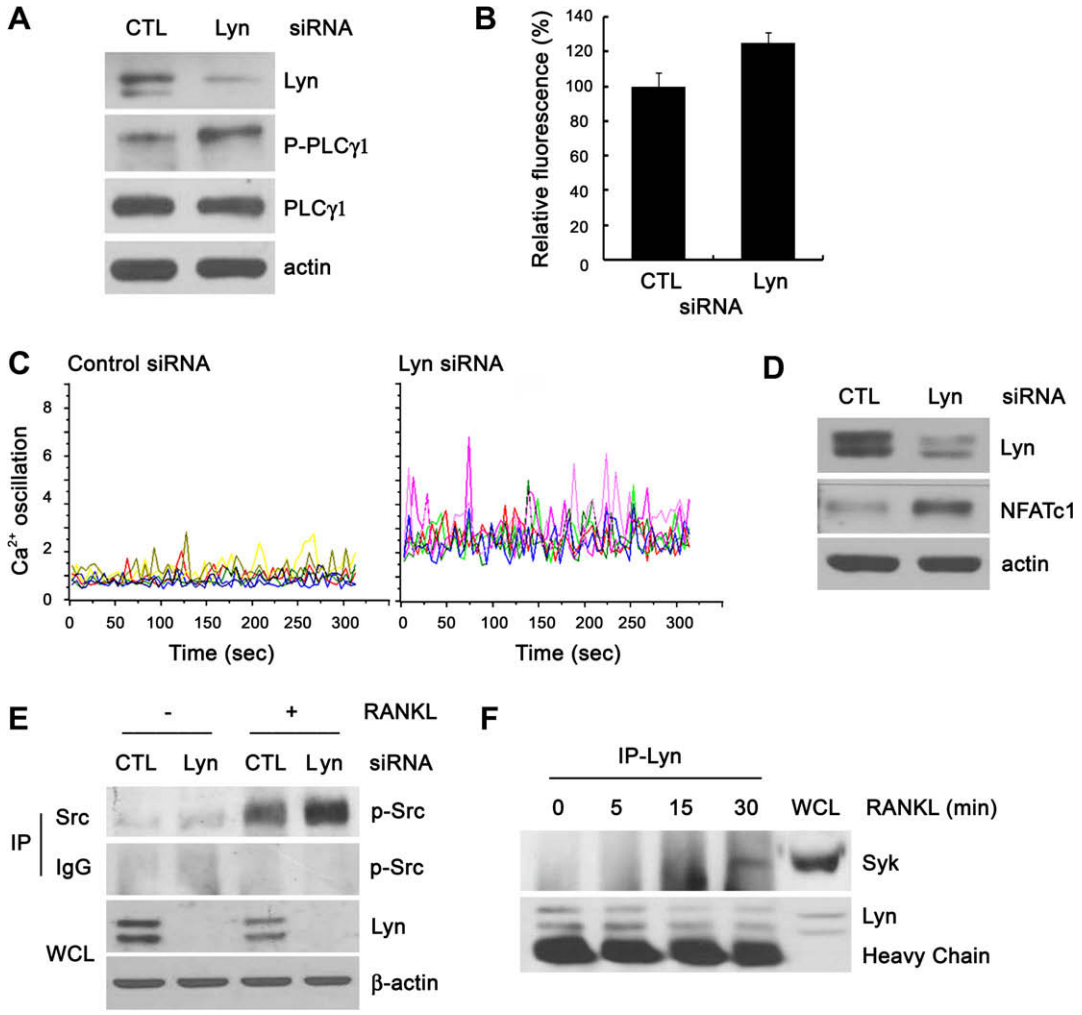

Fig. 3. Inhibition of RANKL-induced PLCγ and Ca^{2+} signaling by Lyn silencing. (A) siRNA-transfected BMMs were cultured with RANKL and M-CSF for 2 days. Cells were serum starved for 5 h and re-stimulated with RANKL (200 ng/ml) for 40 min. Cell lysates were subjected to Western blotting. (B) siRNA-transfected BMMs were seeded in culture plates, incubated with RANKL plus M-CSF for 2 days, and loaded with Fluo-4 NW. The total fluorescence of wells was measured using a plate reader. (C) siRNA-transfected BMMs were placed on cover slips and cultured with RANKL plus M-CSF for 2 days. Cells were loaded with Fura-2/AM and the in situ Ca^{2+} oscillation response of each cell was monitored using a confocal microscope. (D) siRNA-transfected BMMs were cultured with RANKL and M-CSF for 2 days. The levels of NFATc1 and Lyn were determined by Western blotting. (E) siRNA-transfected BMMs were cultured with RANKL and M-CSF for 2 days. Cell lysates were immunoprecipitated with c-Src antibody and Western blotted with phospho-c-Src antibody. (F) BMMs were stimulated with RANKL for the indicated time points. Cell lysates were immunoprecipitated with Lyn antibody and immunoprecipitates were subjected to Western blotting with Syk and Lyn antibodies.

the control siRNA-injected mice (Fig. 4C). These in vivo results uphold the in vitro stimulatory effect of Lyn siRNA on osteoclast differentiation.

4. Discussion

It has been well recognized that RANKL stimulates the activation of intracellular signaling molecules for expression of genes essential for osteoclast differentiation. A few reports indicate that negative regulatory signaling for osteoclastogenesis is also activated upon RANKL stimulation [10]. However, the mechanism of negative regulation is largely unknown. By a proteomics approach using BMMs, we found that the protein level of Lyn was reduced by RANKL treatment. We further characterized the role of Lyn in osteoclast differentiation by gene knock-down. Introduction of Lyn siRNA greatly increased the formation of osteoclasts from primary precursor cells in vitro through a mechanism involving PLCγ and Ca^{2+} oscillation. The pro-osteoclastogenic effect of Lyn siRNA was confirmed in vivo by experiments using a mouse calvarial model of bone resorption. Our results implicate that Lyn is a bona fide negative regulator in RANKL-induced osteoclastogenesis.

It has been reported that Lyn has an inhibitory function in B cell antigen receptor (BCR) signaling by directly phosphorylating Syk at an inhibitory tyrosine residue [3]. Alternatively, Lyn may phosphorylate ITIM motif tyrosines in inhibitory receptors like PIR-B and FcRIIB for recruitment of phosphatases that dephosphorylate Syk and Btk, two key molecules required for BCR signaling [11]. One of the signaling events evoked by BCR is activation of PLCγ by Syk and Btk, and subsequent Ca^{2+} mobilization [11]. Since RANKL also activated PLCγ and Ca^{2+} signals, we postulated that Lyn might participate in RANKL signaling. Our data demonstrated that Lyn gene knock-down in osteoclast precursor cells enhanced PLCγ activation and Ca^{2+} signals, suggesting an analogy present between BCR and RANK signal transduction processes. Indeed, the ITIM-harboring inhibitory receptors LILRB and PIR-B were recently reported to negatively regulate osteoclastogenesis [12]. Whether Lyn-mediated phosphorylation of those ITIM motifs is involved in the inhibition of osteoclastogenesis by Lyn is therefore an intriguing question.

Syk has been implicated for the regulation of osteoclast differentiation. Syk-deficient BMMs fail to differentiate to mature osteoclasts and show defect in bone resorbing activity [13]. Recently, it

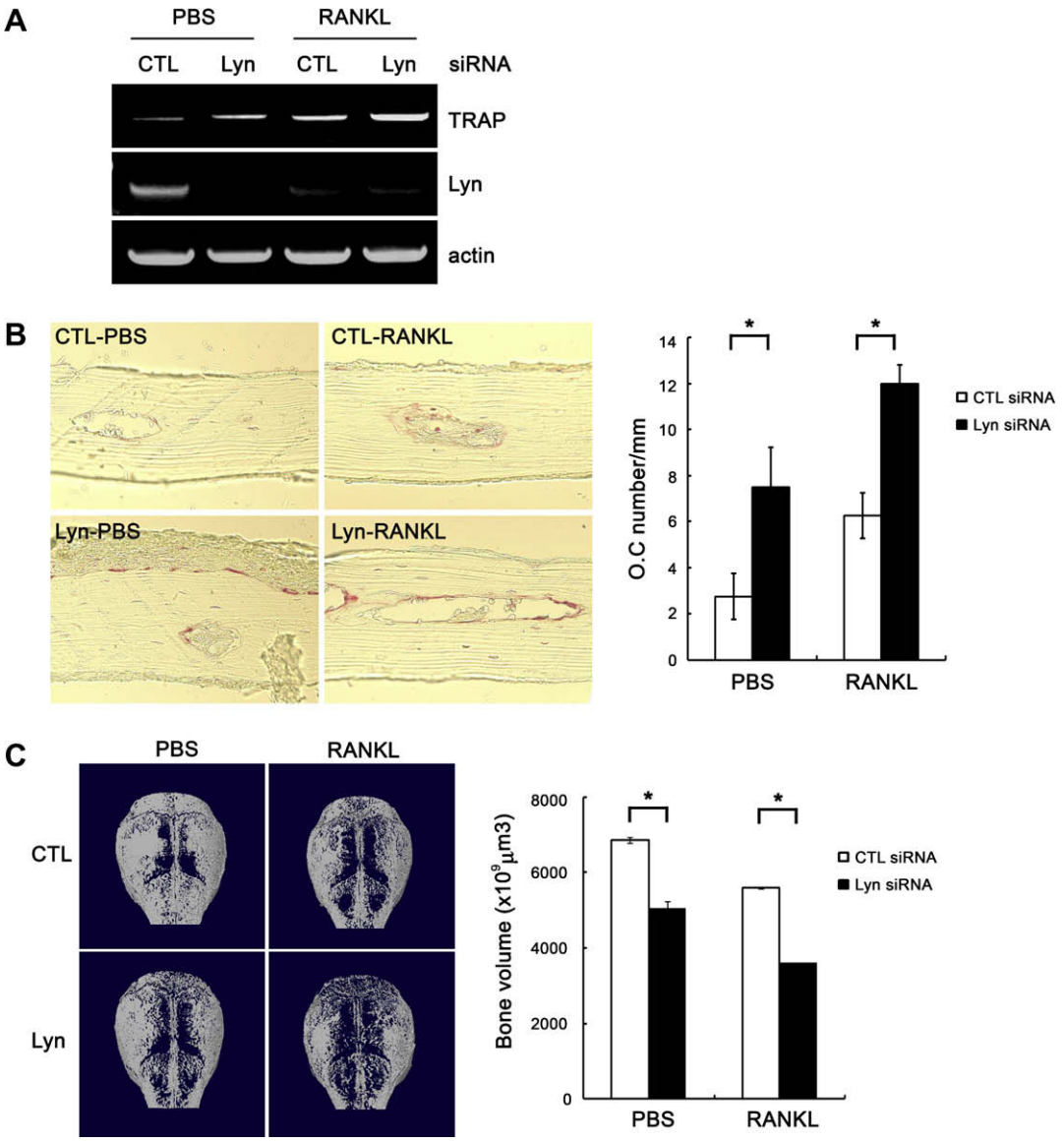

Fig. 4. In vivo effects of Lyn silencing on bone resorption. (A–C) Mice calvariae were injected with Lyn siRNA or scrambled siRNA three times with a 2-day interval. One day after the first injection, mice calvariae were implanted with collagen sheets soaked with RANKL or PBS. mRNA was collected from calvariae and the levels of Lyn and TRAP were determined by RT-PCR (A). Calvariae were decalcified and processed for TRAP staining as described in Materials and methods (B). Undecalcified calvariae were scanned with a μ-CT and analyzed for bone volume (C). $^{*}P < 0.05$ between PBS and RANKL groups and $^{\dagger}P < 0.05$ between control siRNA and Lyn siRNA groups.

is also reported that αvβ3 activation leads to c-Src-mediated Syk phosphorylation and induces osteoclastic bone resorption [9]. We observed that Lyn was co-immunoprecipitated with Syk in BMMs and the interaction was increased by RANKL stimulation. Therefore, it is possible that Lyn inhibits osteoclast differentiation by a mechanism relying on Syk. We also found that Lyn silencing increases phosphorylation of c-Src in BMMs. It is known that Lyn recruit phosphatases such as SHP-1 and SHIP-1 in neutrophils [14]. Thus, a potential mechanism by which Lyn negatively regulates osteoclast differentiation is recruitment of phosphatases to Src and Syk in response to RANKL stimulation. Understanding the sequence of phosphorylation of these kinases by one another and recruitment of phosphatases upon RANKL stimulation requires further investigation.

It has been well established that Lyn play an important role in regulating BCR signaling. Lyn knock-out mice display disrupted B-cell function and defective mast cell degranulation [15,16]. However, skeletal phenotypes of Lyn deficient mice have not been reported and role of Lyn in bone cell regulation has not been investigated. Our in vitro and in vivo data provide the first line of evidence that Lyn has an anti-osteoclastogenic function. Further studies with Lyn knock-out mice may generate corroborative evidence for the involvement of Lyn in osteoclastogenesis regulation. Our study suggests that increasing Lyn activity or expression level may be a useful strategy in developing therapeutics for treating bone lytic diseases.

Acknowledgements

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